Effects of the Booroola gene (*FecB^B*) on bodymass, testis development and hormone concentrations during fetal life

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In female fetuses the Booroola gene (*FecB^B*) is known to affect germ cell development and consequently the pattern of ovarian follicular growth during fetal and post-natal life. However in males, the role of this gene during fetal development is unknown. The aims of the study reported here were to examine the effects of the *FecB^B* gene on development of male fetuses with respect to body and organ mass (for example, pituitary gland, adrenal and mesonephros), testes development, including numbers of germ cells, and also the plasma concentrations or tissue contents of the reproductive hormones (FSH, LH and testosterone) at days 40, 55, 75, 90 and 135 of gestation. The *FecB^B* gene was found to influence litter size, bodymass, crown–rump length and testis mass at most stages of gestation. Some effects were also noted on the mesonephros at days 40 and 55 and on the pituitary and adrenal at days 90 or 135 of gestation. However, the *FecB^B* gene was not observed to have an effect on the patterns of germ cell development, on pituitary content or plasma concentrations of immunoreactive or bioactive FSH or immunoreactive LH or testicular content of testosterone. When embryo transfer experiments were performed to eliminate the effects of litter size at days 40, 90 and 135 of gestation nearly all of the differences in bodymass, crown–rump length and organ mass disappeared. The only exception to this was at day 90 when bodymass continued to be lighter and crown–rump lengths smaller in the BB/B + fetuses compared with the ++ fetuses; the significance of this finding remains unknown. It is concluded that for Booroola male fetuses there are no direct effects of the *FecB^B* gene on pituitary gonadotrophin function or testicular development after sexual differentiation. Moreover, although there may be temporal differences around day 90 of gestation, there are no long-term, direct effects of the *FecB^B* gene on total body, adrenal, testis or pituitary mass. Collectively these findings for the male are similar to those for female fetuses except with regard to germ cell development.

Introduction

The Booroola *FecB^B* gene is manifested in ewes as an increase in ovulation rate (Davis *et al.*, 1982) and is without any obvious effects on the reproductive performance of rams. Nevertheless, *FecB^B*-related differences have been noted in neonatal and adult rams with respect to plasma concentrations of pituitary and gonadal hormones; however, in most, but not all, instances these effects are attributable to their sires rather than to genotype *per se* (Price *et al.*, 1991; Purvis *et al.*, 1991; Isaacs *et al.*, 1992, 1995).

In the female there is clear evidence that the *FecB^B* gene influences ovarian development during fetal, neonatal and adult life, and that these effects are not related to their sires (see McNatty *et al.*, 1995 for review). It is important to note that

the differences in ovarian development in fetal life are not preceded by, or associated with, any *FecB^B* differences in the plasma or pituitary concentrations of gonadotrophin, plasma concentrations of immunoreactive (i) inhibin or the ovarian contents or concentrations of immunoreactive inhibin or steroid. The most consistent effect of the *FecB^B* gene on the ovary during fetal life appears to be related to the timing of germ cell development: at day 40 of gestation more germ cells were found in the ++ genotype than in BB/B + ovaries; at day 55, germ cells had begun to enter meiosis in ++ but not in BB/B + animals; at day 75, primordial follicles were observed in ++ but not in BB/B +; at day 90 more germ cells had been lost in ++ than in BB/B +; and, at day 135 of gestation, there were more secondary or tertiary follicles in ++ than in BB/B + animals. This pattern of retarded gonadal development in the BB/B + genotype was confirmed in embryo-transfer experiments in which litter sizes were balanced. In contrast to the female fetus there are no published data on the effects of

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the *FecB^B* gene on development of the male fetus. There is preliminary evidence to suggest that the *FecB^B* gene may affect postnatal growth. Montgomery *et al.* (1991) compared the growth rates of embryo-transferred BB ($n = 8$) and ++ ($n = 8$) rams born as singletons and found that BB rams grew more slowly from 12 to 64 weeks of age: the mean growth rate difference was 13 g per day, although this did not reach statistical significance at the 5% level.

The aims of the present study were to examine the effects of the *FecB^B* gene on development of male fetuses focusing on measurements of body and organ mass, testes development, including numbers of germ cells, and also, the plasma concentrations or tissue content of the reproductive hormones (FSH, LH and testosterone) at days 40, 55, 75, 90 and 135 of gestation.

Materials and Methods

The experimental procedures reported in this study were carried out in accordance with the 1987 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand after approval was granted by the Animal Ethics Committee of the Wallaceville Animal Research Centre.

Animals and procedures

Male fetuses (BB or B+ and ++) were recovered from pregnant ewes at days 40, 55, 75, 90 and 135 after mating. The BB or B+ male fetuses ($n = 158$) were the results of matings between BB Booroola Romney rams ($n = 5$) and B+ Booroola Romney ewes ($n = 102$) so that all fetuses were either homozygous or heterozygous for the *FecB^B* gene. Since it was not possible at the time of this experiment to distinguish between the BB or B+ fetuses these are hereafter referred to as BB/B+ animals. The ++ male fetuses ($n = 91$) were the result of matings between ++ Booroola Romney rams ($n = 8$) and Romney ewes ($n = 80$). There were no differences in the bodymasses of the BB and ++ rams. Fetuses were recovered after a barbiturate overdose (Euthatal: injection of approximately 20 ml i.v. of a 500 mg ml⁻¹; Chemstock, Palmerston North, New Zealand) was given to their mothers. Fetal blood samples were collected by cardiac puncture, centrifuged (4000 g, 20 min) at 4°C and the plasma stored at -15°C until assayed. Plasma samples were assayed for immunoreactive (i) and bioactive (b) FSH, i-LH and i-inhibin. Bodymasses and crown-rump lengths were measured. The testes, mesonephros (at days 40 and 55 only), adrenals and pituitaries were dissected out and weighed. The left testis was fixed in Bouin's fixative, stained with haematoxylin and eosin, and used for counting germ cells. The right adrenal and right testis were frozen to -70°C for subsequent homogenization and hormone assay.

The entire pituitary gland from each fetus was homogenized into 2 ml buffer (5 mmol potassium phosphate l⁻¹ + 0.1% (w/v) BSA + 1 mmol phenyl methyl sulphonyl fluoride l⁻¹, pH 7.4) with 2 × 25 s bursts in an ultraturrex homogenizer on ice. The homogenates were centrifuged at 400 g for 5 min and then at 30 000 g for 30 min. Thereafter, the supernatants were decanted (extract 1) and the pellets homogenized and centrifuged once more to obtain a second supernatant (extract

2). The supernatants (extract 1 and 2) were stored at -20°C until assayed.

Embryo transfer experiment

The potential effects of unequal litter size at days 40, 90 and 135 of gestation were eliminated by transferring equal numbers of embryos from Booroola Romney BB rams mated to superovulated BB/B+ ewes ($n = 10$) and from Booroola Romney ++ rams mated to superovulated Booroola Romney ++ donor ewes ($n = 10$) to recipient Romney ewes that were neither carriers nor non-carriers of the *FecB^B* gene. The BB/B+ and ++ donor ewes were superovulated using pregnant mares' serum gonadotrophin (PMSG) (Folligon; Intervet, Castle Hill, NSW) and FSH (Ovagen, Immuno-Chemical Products Ltd, Auckland; 20 ml vial is equivalent in potency to 18 mg NIADDK-oFSH-17 as assessed by radioreceptor assay). Briefly BB/B+ and ++ Booroola Romney ewes were fitted with intravaginal progesterone devices (CIDR-s type G; AHI Plastic Moulding Co., Hamilton) for 10 days (day of CIDR-s implant, day 0). On day 10 the CIDR-s device was replaced and the ewes were given an i.m. injection of PMSG (300 i.u.) and 8 × 1 ml injections (i.m.) of a 20 ml ampoule of FSH starting from the time of PMSG injection (morning) and thereafter at intervals of 12 h. The second CIDR-s device was removed on the morning of day 13. On the evening of day 13 and on the morning of day 14 the BB/B+ ewes were mated with BB rams ($n = 6$) and likewise the ++ ewes were mated with ++ rams ($n = 6$). Four days after mating embryos were recovered from the BB/B+ ($n = 144$) and ++ ewes ($n = 102$), respectively. Oestrus-synchronized (that is, with CIDR-s) Romney ewes each received three embryos via a laparoscopic technique. Subsequently 17 BB/B+ and 17 ++ male embryos were recovered from recipient ewes at the equivalent of day 40 of gestation, 24 BB/B+ and 25 ++ males at day 90 and 13 BB/B+ and six ++ males at day 135 gestation. The day 135 embryos were derived from four BB and three ++ superovulated ewes and three sires for each genotype.

Morphometric studies

The volume of the testis (V) was estimated from measuring cross-sectional areas (a) from every 100th section. The volume was estimated using the formula $V = \Sigma ah_{(s)}$ where h is the distance in μm between the sections (s) used to determine a in μm^2 . The number of germ cells was counted from at least 10 pairs of serial sections (each section was 4 μm) per testis using the nuclear dissector method of Gundersen (1986). Briefly, an unbiased counting frame of area $a_{(f)}$ was placed over the projected images of the sections and the number of germ cells exclusive to one section of each pair only (Q) were counted. The total germ cell count (N) was derived from the formula $N = (\Sigma Q / \Sigma a_{(f)}) \cdot h \times V$ where h = distance between the two adjacent sections and V = organ volume. The germ cells were classified as follows: atretic germ cells, where the nucleus was condensed or misshapen and intensely eosinophilic; healthy germ cells, where the nuclear chromatin was uniformly distributed and had a speckled appearance, and premeiotic germ cells where the nuclear chromatin had a 'webb-like' appearance.

Various types of germ cell have been described in the testes (Luciani *et al.*, 1977; de Kretser and Kerr, 1988). The so-called pre-meiotic cells that were observed in the sheep fetal testis appeared coincident with the onset of meiosis of germ cells in the fetal sheep ovary: we therefore concur with Luciani *et al.* (1977) that these cells may be undergoing a preleptotene chromosome condensation stage.

Hormone assays

Immunoreactive-FSH. The radioimmunoassay kit was supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Bethesda, MD. The ovine (o) FSH for iodination was NIAMDD-oFSH-I-1, the oFSH reference preparation was NIDDK-oFSH-RP1 (biopotency $75 \times$ NIH-FSH-S1; Steelman Pohley assay) and the oFSH antiserum was NIDDK-anti-oFSH-1 (AFP-C5288113). For assay, the two supernatants from each pituitary were serially diluted and the results for both added to obtain the total for each pituitary. The results from the serial dilutions ran parallel to the standard curve for all pituitary supernatants. The volume of plasma or pituitary extract used in the assay was 0.1 ml and each diluted sample was assayed in duplicate. The internal standards, standard curve samples and serially diluted extracts were prepared in FSH-free plasma from a hypophysectomized ewe and the minimum detectable concentration was 0.2 ng ml^{-1} . The minimum detectable amount of FSH for the pituitary was $2 \times 10^{-5} \mu\text{g}$ per gland. The intra- and interassay coefficients of variation (CV) for the internal standards were $< 8\%$.

Bioactive-FSH. Plasma and pituitary b-FSH were measured as described by Padmanabhan *et al.* (1987); the only modification was the use of a 0.5 ml substrate incubation volume instead of 1 ml. The validation of this assay for plasma and pituitary extracts from sheep fetuses has been reported by Phillips *et al.* (1992). Concentrations of b-FSH were quantified using the production of oestradiol *in vitro* from Sertoli cells isolated from immature male rats. The reference standard used was NIDDK-oFSH-RP-1. The sensitivity of the oestradiol radioimmunoassay (McNatty *et al.*, 1984) was 5 pg ml^{-1} and the intra- and interassay CVs were both $< 10\%$. The sensitivity of the FSH bioassay was $< 1 \text{ ng ml}^{-1}$ and the intra- and interassay CVs were < 12 and $< 17\%$, respectively.

LH. The radioimmunoassay was identical to that described by McNatty *et al.* (1989). Briefly, the iodination standard was NIADDK-oLH-I-3 (AFP-9598B); the LH antiserum was raised at the Wallaceville Animal Research Centre and is described by McNatty *et al.* (1987). The oLH reference preparation was NIAMDD-oLH-23 (biopotency $2.3 \times$ NIH-LH-S1; ovarian ascorbic acid depletion assay). The volume of pituitary extract or plasma which was assayed in duplicate was 0.1 ml. The internal standard dilutions of pituitary extracts and standard curve samples were prepared in LH-free hypophysectomized ewe plasma. The result from the serial dilutions ran parallel to the standard curve for all pituitary supernatants. The overall intra- and interassay CVs for the internal standards that were included with every standard curve estimation were both $< 10\%$. The minimum detectable concentration of LH was

Table 1. Effect of gestational age and Booroola genotype on litter size, crown-rump length and bodymass in male fetuses

Gestational age in days (genotype)	<i>n</i>	<i>n</i> ₁	Litter size	Crown-rump length (cm)	Bodymass (g)
40 (+ +)	13	11	1.5 (1.2, 1.8)	3.7 (3.6, 3.9)	4.3 (4.0, 4.6)
40 (BB/B +)	25	17	2.9** (2.5, 3.2)	3.7 (3.6, 3.8)	3.9* (3.7, 4.1)
55 (+ +)	30	24	1.9 (1.5, 2.2)	9.1 (8.8, 9.4)	32.0 (29.4, 34.7)
55 (BB/B +)	26	18	2.8** (2.4, 3.2)	8.7* (8.4, 8.9)	27.3** (25.5, 29.2)
75 (+ +)	19	19	1.3 (1.0, 1.5)	17.4 (16.9, 17.8)	208.4 (197.8, 219.7)
75 (BB/B +)	35	23	2.5** (2.2, 2.7)	16.5** (16.2, 16.8)	171.2** (164.8, 177.9)
90 (+ +)	16	14	1.5 (1.2, 1.7)	22.8 (22.1, 23.4)	540.9 (509.0, 575.8)
90 (BB/B +)	41	23	2.8** (2.6, 3.1)	22.1* (21.8, 22.4)	465.0** (447.4, 483.3)
135 (+ +)	13	12	1.5 (1.1, 1.8)	42.6 (41.0, 44.2)	3725 (3380, 4106)
135 (BB/B +)	31	21	2.5** (2.2, 2.8)	36.4** (35.3, 37.5)	2467** (2217, 2747)

Values are geometric means (and 95% confidence limits). *n* = number of ewes; *n*₁ = number of male fetuses; litter size refers to male and female fetuses; **P* < 0.05, ***P* < 0.01 (ANOVA across genotypes at each age).

0.2 ng ml^{-1} . The minimum detectable amount of LH for the pituitary was $2 \times 10^{-5} \mu\text{g}$ per gland.

Testosterone. This steroid was measured as described by McNatty *et al.* (1982). The testosterone antisera (WA-26) was used at a dilution of 1 in 300 (w/v). The gonad or adrenal was homogenized in up to 3 ml of 70% (w/v) methanol, frozen to -20°C overnight and centrifuged at -10°C at 500 *g* for 10 min. The supernatant was decanted into a tube and evaporated to dryness under dry nitrogen gas. Thereafter, the residue was dissolved in 400 μl of assay buffer (0.1 ml phosphate buffer l^{-1} with 0.15 mol NaCl l^{-1} and 0.1% (w/v) gelatin, pH 7.4). The detection limit was 100 pg ml^{-1} . The inter- and intra-assay coefficients of variation were $< 12\%$.

Statistical analyses

Data from individual fetuses were log-transformed and subjected to analysis of variance to test for differences between genotypes. When the variances between the data sets were unequal, genotype differences were examined using the Kruskal Wallis test. Differences between genotypes were confirmed using partial correlation analyses controlled for litter size. The possibility of differences arising from the use of Booroola Romney and Romney control animals (that is, as the ++ animals) was tested by analysis of variance using breed (Booroola Romney or Romney), gestational age and gonadal characteristic or hormone concentrations as the variables; no

Table 2. Effect of gestational age and Booroola genotype on masses of mesonephros, pituitary, testis and adrenal in male fetuses

Gestational age in days (genotype)	<i>n</i>	<i>n</i> ₁	Mesonephros	Pituitary (mg)	Testis (mg)	Adrenal (mg)
40 (++)	13	11	17 (15, 20)	—	3.4 (3.2, 3.9)	1.6 (1.4, 1.8)
40 (BB/B+)	25	17	14* (12, 16)	—	3.0** (2.7, 3.2)	1.8 (1.4, 2.1)
55 (++)	30	24	17 (15, 19)	5.8 (4.4, 7.4)	15.5 (13.2, 18.1)	13 (11, 15)
55 (BB/B+)	26	16	13** (10, 15)	4.6 (3.9, 5.5)	13.2 (9.9, 16.5)	11 (10, 13)
75 (++)	19	19	—	20 (10, 30)	49.1 (41.8, 56.3)	37 (33, 41)
75 (BB/B+)	35	23	—	13 (12, 14)	35.6** (32.0, 39.1)	33 (30, 37)
90 (++)	16	14	—	31 (26, 36)	105 (96, 114)	59 (54, 63)
90 (BB/B+)	41	23	—	25* (22, 28)	83** (77, 90)	52 (48, 57)
135 (++)	12	11	—	88 (73, 105)	686 (586, 837)	203 (173, 23)
135 (BB/B+)	31	20	—	76 (68, 83)	448** (401, 496)	151** (138, 161)

Values are geometric means (and 95% confidence limits). Masses for testis and adrenal are those for the left organ only. *n* = number of ewes; *n*₁ = number of male fetuses, **P* < 0.05, ***P* < 0.01 (ANOVA across genotypes at each age).

differences were noted. Partial correlation analyses controlled for genotype were performed to determine the effects of litter size. These effects were also confirmed by multiple regression analyses. The data are presented as geometric means (and 95% confidence limits).

Results

Effects of gestational age and Booroola genotype on litter size, crown-rump length, bodymass and organ mass

There were increases in crown-rump length and bodymass and also in pituitary, testis and adrenal masses during gestation irrespective of genotype (Tables 1 and 2). There were significant effects of genotype on litter size at every gestational age (all *P* < 0.01). There were significant effects of genotype at every gestational age for crown-rump length and bodymass except at day 40 of gestation for crown-rump length, with the BB/B+ fetuses being consistently smaller and lighter. Partial correlation analyses controlled for genotype detected significant effects of litter size on crown-rump length at day 135 (*P* < 0.05) and on bodymass at day 90 (*P* < 0.05) and day 135 (*P* < 0.01), but not at earlier ages.

For the mesonephros, the mean masses in the BB/B+ genotype were also consistently lighter than in the ++ animals at days 40 and 55 of gestation. Partial correlation analysis controlled for genotype revealed a significant effect of litter size on the mass of mesonephros at day 40 (*P* < 0.05) but not at day 55 of gestation. With regard to the pituitary and

adrenal glands, no significant effects of genotype were noted at any gestational age except for the pituitary at day 90 and for the adrenals at day 135. Partial correlation analyses revealed significant effects of litter size on the pituitary gland at days 90 and 135 (both *P* < 0.05); no effects of litter size were noted on the adrenals at any gestational age. Mean masses of testes in the BB/B+ genotype were consistently lighter than in the ++ animals throughout gestation; these differences were significant at days 40, 75, 90 and 135 gestation. Partial correlation analyses controlled for genotypes revealed no significant effects of litter size on testes mass at any gestational age.

Effect of gestational age and Booroola genotype on testis characteristics during fetal development

The data for testis volume, total number of germ cells, number of atretic germ cells, number of healthy germ cells and number of premeiotic germ cells are summarized with respect to Booroola genotype and day of gestation (Table 3). The morphological features of atretic, healthy and premeiotic germ cells are shown (Fig. 1). Mean testis volume increased throughout gestation irrespective of genotype. No significant effects of genotype were noted at any gestational age. Partial correlation analyses controlled for genotype revealed that there were no significant effects of litter size on testis volume at any gestational age.

The total number of germ cells increased during gestation in ++ and BB/B+ fetuses. No effects of genotype on total

Table 3. Effect of gestational age and Booroola genotype on testis volume and number of germ cells during development of male fetuses

Gestational age in days (genotype)	n_1	n	Testis volume (μm^3)	Number ($\times 10^{-4}$) of germ cells			Total number ($\times 10^{-4}$) of germ cells
				Atretic cells	Healthy cells	Premeiotic cells	
40 (++)	7	8	1.2 (0.9, 1.5)	—	3.6 (2.3, 5.7)	—	3.6 (2.3, 5.7)
40 (BB/B+)	5	6	1.3 (1.0, 1.6)	—	3.5 (2.1, 5.6)	—	3.5 (2.1, 5.6)
55 (++)	8	10	6.6 (5.9, 7.3)	—	11.5 (9.6, 14.0)	—	11.5 (9.6, 14.0)
55 (BB/B+)	6	8	6.0 (4.8, 7.3)	—	13.0 (10.0, 16.0)	—	13.0 (10.0, 16.0)
75 (++)	7	7	23.6 (13.9, 39.6)	45.4 (20.1, 102.5)	37.3 (20.3, 68.6)	2.7 (0.1, 424.2)	108.1 (63.1, 185.2)
75 (BB/B+)	3	7	15.6 (9.4, 22.4)	23.8 (9.3, 61.1)	38.4 (25.4, 58.1)	1.9 (0.1, 112.7)	80.3 (53.6, 120.3)
90 (++)	8	9	54.1 (32.9, 88.6)	197.5 (142.2, 274.2)	72.7 (54.9, 96.2)	64.4 (45.2, 91.9)	359.8 (297.6, 435.2)
90 (BB/B+)	10	12	48.4 (35.3, 66.3)	88.0 (58.4, 132.5)	53.0 (30.2, 93.0)	35.0 (22.7, 54.1)	204.5 (151.2, 276.9)
135 (++)	8	9	264.8 (215.9, 324.6)	—	429.7 (269.3, 685.5)	—	429.7 (269.3, 385.5)
135 (BB/B+)	7	10	290.0 (237.1, 355.3)	—	457.8 (350.4, 588.2)	—	457.8 (356.4, 588.2)

Values are geometric means (and 95% confidence limits). Number of germ cells are those for one testis. n = number of ewes; n_1 = number of male fetuses, ANOVA across genotypes at each age revealed no significant differences for each variable.

Fig. 1. Photomicrograph showing examples of atretic (a), healthy (h) and premeiotic (pm) germ cells in a testis from a sheep fetus at day 90 of gestation. Scale bar represents 10 μm .

number of germ cells were noted at any age. Atretic germ cells were not seen in appreciable numbers at days 40 and 55 of gestation. However, they were present in large numbers at days 75 and 90 with between 20 and 60% of all germ cells being atretic at these times. No genotype effects were noted. The number of healthy germ cells increased throughout gestation reaching about 5×10^6 at day 135 of gestation. No effect of Booroola genotype was noted at any age. Premeiotic germ cells were observed only at days 75 and 90 with the maximum

number at day 90 in both genotypes. No significant effects of genotype were noted.

Partial correlation analyses controlled for genotype showed no significant effects of litter size on total number of germ cells or number of atretic, healthy or premeiotic germ cells at any gestational age.

Effect of Booroola genotype on fetal characteristics in embryo-transferred ewes at days 40, 90 or 135 of gestation

The purpose of the embryo transfer experiment at day 40 was to establish whether the genotype effects on bodymass and testis mass could be confirmed when litter sizes between the genotypes were not different. At day 40, the geometric means (and 95% confidence limits) for litter size for the BB and ++ embryos were 2.4 (1.9, 2.9) and 2.4 (1.8, 3.1), respectively. The geometric means (and 95% confidence limits) for the bodymasses, testis masses and mesonephros of the BB/B+ ($n = 17$) and ++ ($n = 17$) embryos were: 4.1 (3.8, 4.4) and 4.3 (3.9, 4.6) g; 2.8 (2.5, 3.0) and 3.0 (2.5, 3.7) mg, and 15 (13, 16) and 14 (12, 16) mg, respectively; none of these means was significantly different with respect to genotype. No genotype differences were noted as regards to the other measurements made at day 40 (that is, adrenal mass, crown-rump length, total number of germ cells).

The purpose of the embryo-transfer experiments at days 90 and 135 was to examine the effects of genotype on a wide range of fetal characteristics including crown-rump length,

Table 4. Effect of Booroola genotype on fetal characteristics in embryo-transferred Booroola male fetuses at day 90 of gestation

Fetal characteristics	Booroola genotype	
	++	BB/B +
Litter size	2.3 (1.9, 2.7)	2.1 (1.4, 3.0)
Crown–rump length (cm)	24.2* (23.8, 24.7)	23.3 (22.9, 23.9)
Bodymass (g)	500.3* (504.1, 601.8)	510.7 (485.0, 537.6)
Pituitary mass (mg)	29 (26, 33)	33 (30, 37)
Testis mass (mg)	99.5 (92.4, 106.6)	110.0 (101.1, 120.6)
Adrenal mass (mg)	65.4 (58.5, 72.3)	56.3 (52.0, 60.1)
[†] Testis volume (mm ³)	55.7 (45.8, 84.1)	57.0 (43.9, 74.2)
[†] Number of germ cells (× 10 ^{−4})	176.6 (115.4, 270.4)	118.4 (93.2, 150.4)
<i>n</i>	16	24
[†] <i>n</i> ₁	7	6

Values are geometric means (and 95% confidence limits). *n* = number of male fetuses; [†]*n*₁ = number of fetuses used for germ cell counting and measurement of testis volume. The mean testis masses, volumes and number of germ cells are all from the left gland. **P* < 0.05 (Kruskal–Wallis test).

bodymass, pituitary mass and testis volume when the litter sizes were equal. A summary of the characteristics that were compared at day 90 is shown (Table 4). At day 90, genotype differences were noted in the mean crown–rump length, and mean bodymasses, but not in litter size, testis mass, testis volume, pituitary mass, adrenal mass or number of germ cells. A summary of the characteristics that were compared at day 135 is shown (Table 5); no genotype differences were noted for any of the fetal characteristics that were examined.

Changes in the plasma concentrations and pituitary contents of b-FSH, i-FSH and i-LH with respect to day of gestation and genotype

The plasma and pituitary contents of FSH or LH were not detectable at day 40 of gestation. Both hormones were detectable by radioimmunoassay from day 55 in both plasma and the pituitary gland. Bioactive FSH was not assessed in plasma at day 55 but it was measured in the pituitary gland where activity was detected. No genotype effects were noted for b-FSH, i-FSH or i-LH at any gestational age and no effects of litter size were observed. Accordingly all the data were pooled over the genotypes for each gestational age and the result summarized for b-FSH and i-FSH (Fig. 2) and for i-LH (Fig. 3).

Peak values were noted for plasma i-FSH at day 90, whereas at other ages (days 55, 75 and 135) mean values were similar. No differences were noted for b-FSH with respect to

Table 5. Effect of Booroola genotype on fetal characteristics in embryo-transferred Booroola male fetuses at day 135 of gestation

Fetal characteristics	Booroola genotype	
	++	BB/B +
Litter size	2.6 (2.1, 3.1)	1.9 (1.6, 2.3)
Crown–rump length (cm)	39.1 (35.7, 42.8)	40.5 (39.5, 41.6)
Bodymass (g)	2780 (2214, 3491)	3101 (2876, 3344)
Pituitary mass (mg)	68 (56, 80)	80 (71, 89)
Testis mass (mg)	450 (310, 620)	540 (500, 590)
Adrenal mass (mg)	420 (360, 480)	460 (420, 500)
[†] Testis volume (mm ³)	432 (322, 579)	592 (528, 664)
[†] Number of germ cells (× 10 ^{−4})	570.3 (379.9, 856.1)	745.1 (606.4, 915.4)
<i>n</i>	6	13
[†] <i>n</i> ₁	6	7

Values are geometric means (and 95% confidence limits). *n* = number of male fetuses; [†]*n*₁ = number of fetuses used for germ cell counting and measurement of testis volume. The mean masses, volumes and number of germ cells are all from the left gland.

gestational age. The contents of pituitary i-FSH and b-FSH increased throughout gestation.

The mean concentrations of plasma i-LH declined from day 75 to relatively low values at day 135, whereas they increased throughout gestation in the pituitary gland.

Changes in testosterone content

No genotype effects at any age were noted for testicular or adrenal content of testosterone and no effects of litter size were noted. Accordingly the results for the genotypes were pooled with respect to gestational age (Fig. 4). Testosterone was detected in both organs from day 55 to 135; no measurements were made at day 40. The mean testosterone content of the testes was always higher than that in the adrenals. The highest testosterone content in the testis was noted at days 55 and 75; thereafter, values declined significantly to approximately 700 pg per gland at day 135. Testosterone content of the adrenals did not change between days 55 and 90; however, by day 135, they had increased around twofold (*P* < 0.01).

Discussion

The results presented here show that for male fetuses the *FecB*^B gene influences litter size, bodymass, crown–rump length and testis mass at most stages of gestation. Some effects of the *FecB*^B gene were also noted on the masses of the mesonephros at days 40 and 55 and of the pituitary and adrenal glands at

Fig. 2. Plasma concentrations of (a) immunoreactive FSH (i-FSH), (b) bioactive FSH (b-FSH) and pituitary contents of (c) i-FSH and (d) b-FSH in male BB/B+ and ++ (pooled data) Booroola fetuses at different days of gestation. Histograms represent geometric means and vertical bars are the 95% confidence limits. Numbers in parentheses refer to the number of fetuses. Histograms with different letters are significantly different ($P < 0.05$).

days 90 or 135 of gestation. However, no effects of the *FecB*^B gene were found for germ cell development, testicular testosterone content, pituitary gonadotrophin content or pituitary hormone concentrations. Moreover, although not reported here, no differences were noted for the concentrations of inhibin in plasma or the contents of testicular inhibin using an inhibin assay kit (supplied by the National Institute of Child Health and Human Development, USA). The forms of inhibin present in male fetuses have not been characterized, and the inhibin antiserum used is unlikely to distinguish the intact molecule from some forms of the α -subunit. Thus the role of inhibin in fetal development requires further study. A key issue in elucidating the role of the *FecB*^B gene in fetal development is to establish whether any of the above differences are due directly to genotype or indirectly as a consequence of litter size, sire effects or other factors, such as the maternal intra-uterine environment. The embryo transfer experiments at days 40, 90 and 135 eliminated the maternal intrauterine and litter size effects. They also resolved any potential uncertainties in the main study where the ++ male fetuses were derived from

Booroola Romney sires and Romney ewes. As a result, all the *FecB*^B-associated effects on bodymass, crown-rump length and mass of mesonephros, pituitary, testis and adrenal disappeared. The only exception to this was at day 90 when bodymass was lighter and the mean crown-rump length was smaller in the BB/B+ fetuses compared with the ++ fetuses. The significance of this finding remains obscure as no differences were noted at days 40 or 135 or at other ages (for example, days 95, 100, 120) that were not reported in this study. It is of importance that, in the embryo transfer experiments, no differences between the genotypes were noted in the numbers of germ cells, a finding consistent with the observations in the main study.

In rats, it has been shown that the presence of a male neighbour may alter sexual development of female fetuses (Vom Saal *et al.*, 1991). The possibility that the number of adjacent female or male fetuses influences the growth characteristics (that is, crown-rump length, bodymass) of the male fetuses was examined; however, no obvious effects were noted. The effect of the sire is known to be related postnatally to

Fig. 3. Plasma concentrations of (a) immunoreactive LH (i-LH) and pituitary contents of (b) i-LH in BB/B+ and ++ (pooled data) male Booroola fetuses at different days of gestation. Histograms represent geometric means and vertical bars are the 95% confidence limits. Numbers in parentheses refer to the number of fetuses. Histograms with different letters are significantly different ($P < 0.05$).

plasma concentrations of i-FSH and inhibin (Price *et al.*, 1991; Purvis *et al.*, 1991; Isaacs *et al.*, 1992). In the present study, it was not possible to study the effects of sire for each genotype at every gestational age. However, in the embryo-transfer experiments at day 135, single-sire matings were performed and the effects of sire could be examined: at this fetal age, no effects of sire were noted.

It is of interest to compare the present results for male fetuses with those previously reported for female fetuses (Smith *et al.*, 1993, 1994; McNatty *et al.*, 1995). In the female, *FecB*^B differences in bodymass, independent of litter size, were observed at days 35 and 40, but not at days 28, 30, 55, 75, 90, 100, 120 or 135. At days 35 and 40, BB/B+ fetuses were found to be lighter than the ++ genotype without any differences noted in crown-rump length. Also in the female no

Fig. 4. Testosterone content of testis (□) and adrenal (■) glands in BB/B+ (pooled data) male Booroola fetuses at different days of gestation. Contents were determined in the right-hand organ only. Histograms represent geometric means and vertical bars are the 95% confidence limits. Numbers in parentheses refer to the number of fetuses. Histograms with different letters are significantly different ($P < 0.05$) (with respect to the different organs).

consistent differences were noted in ovarian volume or mass, adrenal mass or pituitary mass between the genotypes. In the male, the embryo-transfer studies revealed that when litter size is balanced *FecB*^B differences in bodymass or crown-rump length occur only at day 90 but not at day 40 or days 95–135. Collectively these results suggest that, if there are any genotype differences in bodymass or organ mass or volume, they tend to be a consequence of litter size rather than directly of genotype. In the female fetuses, unlike the male, germ cell development is retarded in the BB/B+ genotype. Fewer oogonia (days 30–40), primordial follicles (days 75–90) and growing follicles (day 120 to 6 weeks after birth) are noted in those carrying the *FecB*^B gene (McNatty *et al.*, 1995). In contrast, in the male, the number of healthy germ cells, atretic germ cells or premeiotic germ cells are not different between the genotypes.

In both males and females, the *FecB*^B gene is not associated with differences in plasma gonadotrophin concentrations during fetal life. In postnatal or adult life there is no unequivocal evidence to suggest that the *FecB*^B gene results in differences with respect to gonadotrophin concentrations in plasma in males. Indeed it would appear that most differences, if present, are a consequence of sire. In conclusion, we propose, on the basis of our results, that in Booroola males there are no long-term, direct effects of the *FecB*^B gene on bodymass or organ or germ cell development after sexual differentiation. Whether there are *FecB*^B differences in the male genotype earlier in embryonic life remains to be determined.

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